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Laminin and Integrin Expression in the Ventral Ectodermal Ridge of the Mouse Embryo: Implications for Regulation of BMP Signalling

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Background: The ventral ectodermal ridge (VER) is an important signalling centre in the mouse tail-bud following completion of gastrulation. BMP regulation is essential for VER function, but how these signals are transmitted between adjacent tissues is unclear. Results: We investigated the idea that extracellular matrix components might be involved, using immunohistochemistry and in situ hybridisation to detect all known α, β, and γ laminin chains and their mRNAs in the early tail bud. We identified an apparently novel laminin variant, comprising α5, β3 and γ2 chains, as a major component of the VER basement membrane at E9.5. Strikingly, only the mRNAs for these chains were co-expressed in VER cells, suggesting that lamin532 may be the sole basement membrane laminin at this stage. Since α6 integrin was also expressed in VER cells, this raises the possibility of cell-matrix interactions regulating BMP signalling at this site of caudal morphogenesis. Conclusions: Laminin532 could interact with α6-containing integrin to direct differentiation of the specialised VER cells from surface ectoderm. *Developmental Dynamics 241:1808–1815, 2012.* © 2012 Wiley Periodicals, Inc.

Key words: mouse; embryo; tail bud; ventral ectodermal ridge; laminin; extracellular matrix; basement membrane; integrin; bone morphogenetic protein

Key findings

- Laminin α5, β3, and γ2 chains are co-expressed by cells of the ventral ectodermal ridge (VER) in the E9.5 mouse embryo.
- A possible new laminin532 variant is expressed by VER cells.
- Laminin 532 may interact with α6-containing integrins to mediate BMP signalling during development of the mouse tail bud.

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INTRODUCTION

After completion of gastrulation, the tail begins to form in the caudal-most region of the mouse embryo, beginning with the generation of the tail-bud. From embryonic day (E) 9.5 onwards, the tail-bud contains a morphologically distinct group of ectodermal cells known as the ventral ectodermal ridge (VER). The VER is a source of signals that regulate tail

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development, and contains progenitor cells that contribute to the ventral midline ectoderm of the tail (Gruneberg, 1956; Goldman et al., 2000). Ablation of the VER leads to defects in somitogenesis and tail elongation (Goldman et al., 2000). However, the mechanisms through which the VER exerts its effect on these processes have not been fully elucidated.

One candidate player in mediating the effects of the VER is the bone morphogenetic protein (BMP), Bmp2. Bmp2 is expressed in the cells of the VER, while the BMP antagonist, noggin, is expressed in the immediately adjacent mesoderm of the ventral tail-bud. Noggin expression is no longer detected in the ventral mesoderm after ablation of the VER (Goldman et al., 2000). Although the signals from the VER that regulate noggin expression are unclear, it is suggested that Bmp2 could induce noggin production, as part of a negative feedback loop (Goldman et al., 2000). The signals that control the restricted expression of Bmp2 in the VER have yet to be identified.

The BMPs form a large subclass of the transforming growth factor β (TGFβ) superfamily of signalling molecules, with demonstrated involvement in embryonic events including neurulation and dorso-ventral patterning (Mehler et al., 1997). BMPs have been specifically implicated in regulating outgrowth and patterning of the *Xenopus* tail-bud (Beck et al., 2001) and, together with nodal, in function of the zebrafish tail organizer (Agathon et al., 2003; Fauny et al., 2009). The BMP signalling pathway is well characterised (Attisano and Wrana, 2002), and its activity can be monitored by analyzing the expression of downstream genes such as *Cadherin6, Rhob* (Sela-Donenfeld and Kalcheim, 1999), and *Msx1* and *Msx2* (Marazzi et al., 1997; Suzuki et al., 1997; Kettunen and Thesleff, 1998).

BMP signalling is regulated by extracellular antagonists including chordin, chordin-like 1 (Chrdl1; also called neuralin1), follistatin, and noggin, and by the intracellular antagonists Smad6 and Smad7 (Attisano and Wrana, 2002; Rider and Mulloy, 2010). We previously described how Bmp2 signalling is modulated by its antagonists, and by sonic hedgehog (Shh), during the process of spinal neural tube closure (Ybot-Gonzalez et al., 2007). In addition to these well-described BMP regulators, other factors, such as the extracellular matrix components collagen IV, heparan sulphate proteoglycans, and laminins, have also been found to play a role in modulating BMP signalling (Belenkaya et al., 2004; Wang et al., 2008; Dolez et al., 2011). It is unclear whether any of these extracellular modulators are involved in the regulation of BMP signalling in the VER.

One group of potential extracellular modulators of BMP signalling are the laminins, which are major glycoprotein components of basement membranes. Laminins have been implicated in many biological processes, including cell adhesion, migration, and differentiation (Colognato and Yurchenco, 2000; Miner and Yurchenco, 2004). At least 16 different laminin variants exist, and their expression in basement membranes is spatially and developmentally regulated (Tunggal et al., 2000; Yurchenco et al., 2004; Aumailley et al., 2005; Tzu and Marinkovich, 2008). Laminins are heterotrimers containing an α, β, and γ chain in a cross-like three-dimensional structure (Colognato and Yurchenco, 2000). To date, five distinct α chains, three β chains, and three γ chains have been described, and their various combinations define the different laminin isoforms (Miner et al., 1997; Patton et al., 1997; Miner and Yurchenco, 2004). Basement membranes can contain more than one laminin isoform (Yurchenco et al., 2004; Miner, 2008) but, owing to the intracellular assembly of the laminin heterotrimer prior to its secretion, co-expression of α, β, and γ chain mRNAs in a particular cell type is obligatory for production of each specific laminin isoform.

Cellular responses to laminin are determined in part by a group of transmembrane receptors known as integrins (Miranti and Brugge, 2002). The integrin family is composed of 24 α, β heterodimeric members that mediate the attachment of cells to the extracellular matrix (Barczyk et al., 2010). Integrins containing the α3 and α6 subunits have been described as receptors for laminin, regulating activities such as organization of the basement membrane and differentiation of several epithelial cell types (Sorokin et al., 1990; Kadoya et al., 1995; Walker and Menko, 1999). Interestingly, during osteoblast differentiation, Bmp2 has been reported to stimulate the expression of αV and β integrins, which, in turn, are essential for Bmp2 activity (Lai and Cheng, 2005).

In an effort to gain insight into the factors controlling Bmp2 signalling in the VER, we have studied the mRNA expression of Bmp2 signalling components, together with the protein and mRNA expression patterns of all known laminin chains, in the tail-bud of the mouse embryo. We also examined expression of the α3 and α6 integrin subunits. Taken together, our results suggest the existence of a previously undescribed laminin variant that may be implicated in the regulation of Bmp2 responsiveness in the VER via interaction with α6-containing integrin.

RESULTS AND DISCUSSION

Expression of BMP Signalling Components

Whole mount in situ hybridisation for *Bmp2* in mouse embryos at E9.5 revealed intense mRNA expression, specifically within the VER (Fig. 1a–c). We asked whether this strong expression of *Bmp2* might correlate with activation of the BMP signalling pathway in the vicinity of the VER. The BMP downstream genes *Rhob, Msx1*, and *Msx2* were all expressed in the ventral mesoderm overlying the VER (Fig. 1d–f). In contrast, *Cdh6* (Cadherin 6) was not expressed either in the VER or in the surrounding mesoderm (data not shown and Ybot-Gonzalez et al., 2007). While *Msx2* was also expressed in the surface ectoderm, including the VER itself, *Msx1* and *Rhob* transcripts were strikingly excluded from this region.

Bmp2 signalling can be modulated by specific extracellular inhibitors: for example, noggin, which is directly implicated in the development of the tail-bud and VER (Goldman et al., 2000). We detected noggin expression in the mesoderm adjacent to the lateral aspects of the VER (Fig. 1g). In

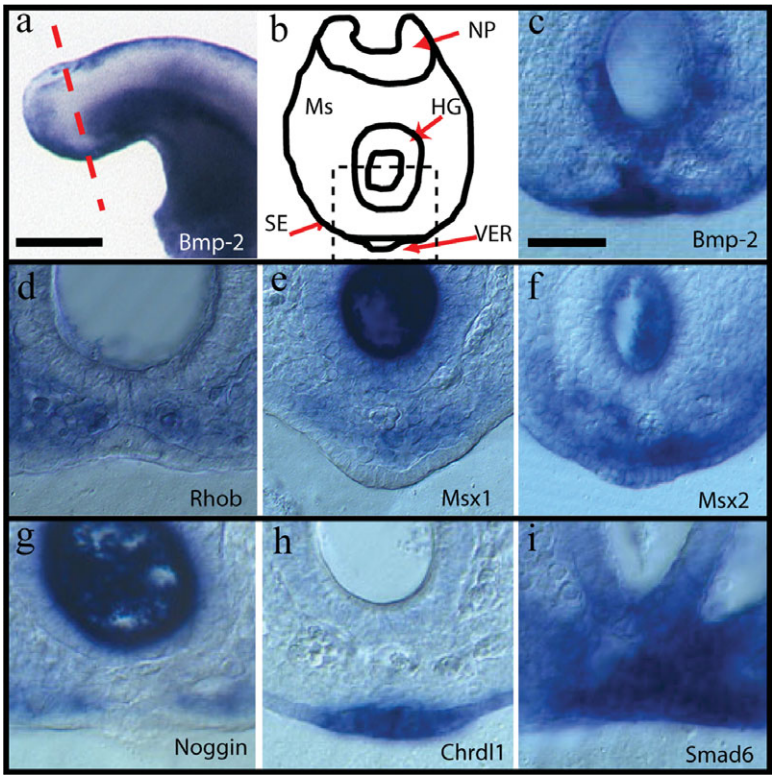

Fig. 1. Expression of genes in the Bmp2 signalling pathway as detected by in situ hybridisation. a: Expression of *Bmp2* in the tail-bud of an E9.5 (25–30 somites) mouse embryo. b: Diagram of the transverse section represented in a by the red dashed line. The black dashed square represents the area of this section shown at higher magnification in c–i and in Figures 2–4. The main tissues are indicated: HG, hindgut; Ms, mesenchyme; NP, neural plate; SE, surface ectoderm; VER, ventral ectodermal ridge. c–i: mRNA distribution on 50-μm vibratome sections following whole mount in situ hybridisation of embryos with individual probes for: *Bmp2* (c), *Rhob* (d), *Msx1* (e), *Msx2* (f), *noggin* (g), *Chrdl1* (*neuralin1*) (h), and *Smad6* (i). Scale bar in a = 0.3 mm and in c= 50 μm (also applies to all other panels).

previous studies, we demonstrated that beads coated in Bmp2 could induce the expression of noggin in surrounding tissues (Ybot-Gonzalez et al., 2007). Hence, the *noggin* expression in the mesoderm may be induced by Bmp2 activity derived from the VER. At later stages of tail-bud development, once neural tube closure is complete, noggin expression is necessary for suppression of epithelio-mesenchymal transition as gastrulation is completed (Ohta et al., 2007). Interestingly, in our study, in addition to *noggin* expression at the early tail-bud stages analyzed, we also detected expression of other BMP inhibitors *Chrdl1* (neuralin 1) and *Smad6*. In contrast to noggin, *Chrdl1* was

Fig. 2. Expression of laminin chain mRNAs and proteins at the VER and surrounding tissues. a–k: Laminins α1–5, β1–3, and γ1–3 mRNA expression on vibratome sections following whole mount in situ hybridisation in individual mouse embryos at E9.5 (25–30 somites). a′–k′: Protein immunolocalization for laminins α1–5, β1–3, and γ1–3 on cryosections of individual E9.5 mouse embryo tail-buds. Arrowheads point to specific labelling in the VER. Asterisks in c′ indicate laminin α3 chain in the basement membrane of the surface ectoderm on either side of (but not overlying) the VER. Scale bar in e = 50 μm (also applies to all other panels).

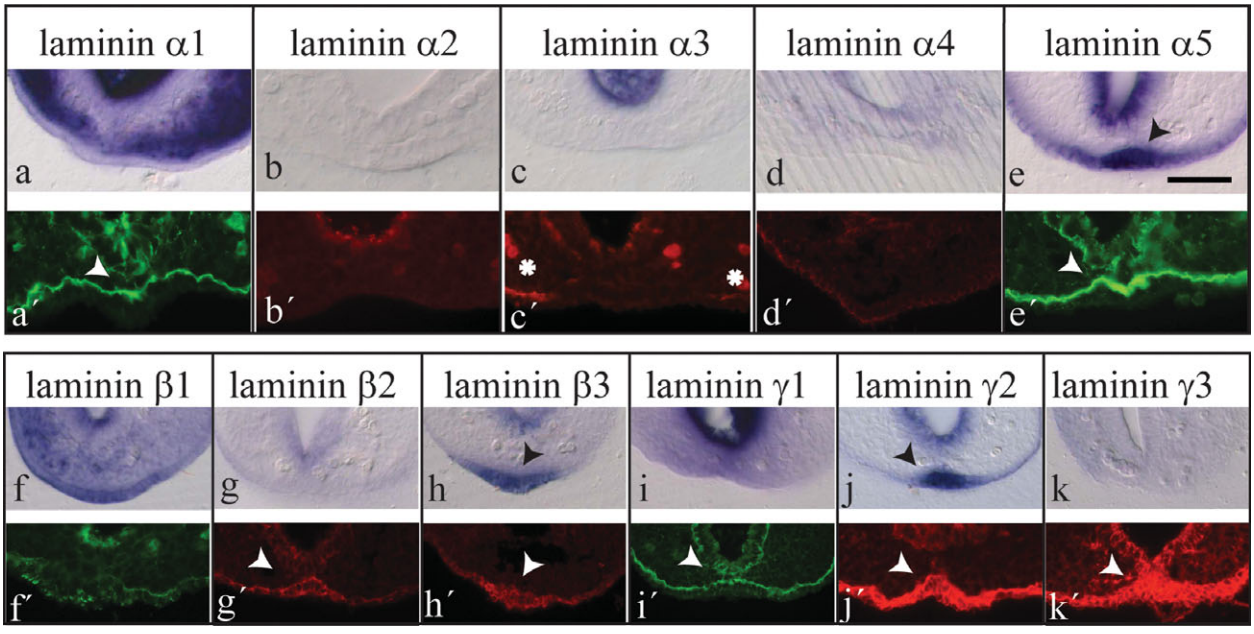

Fig. 2.

expressed specifically in the VER (Fig. 1h), and *Smad6* was expressed in both VER and overlying mesoderm (Fig. 1i). We conclude that BMP signalling is active in the vicinity of the VER, and is controlled by a complex and highly regulated system of agonists and antagonists expressed in the surface ectoderm and adjacent mesenchyme.

Expression of Specific Laminin Isoforms

There is increasing evidence that diffusible morphogens such as Bmp2 are modulated in their activity by interacting with components of the extracellular matrix (Belenkaya et al., 2004; Lai and Cheng, 2005; Wang et al., 2008; Dolez et al., 2011). Hence, a complete understanding of the role of BMP signalling in development of the tail-bud requires an analysis of matrix components. We, therefore, performed in situ hybridisation and immunohistochemistry to detect mRNA and protein for all of the known laminin isoforms in the VER region.

Among the α chains, laminins α1 and α5 were detectable at the protein level in the basement membrane underlying the VER (Fig. 2a', e'), while laminin α3 protein was present at the edges of the VER (Fig. 2c'). Laminin proteins α2 and α4 were not detected in the vicinity of the VER (Fig. 2b', d'). Interestingly, the mRNAs for laminins α1 and α5 exhibit very different spatial distributions. While α1 mRNA is expressed solely in the mesoderm of the ventral tail bud (Fig. 2a), α5 mRNA has an entirely complementary distribution, with expression in the VER and adjacent surface ectoderm, but not in the mesoderm (Fig. 2e). Laminin α2, α3, and α4 mRNAs were not detectable in either mesenchyme or surface ectoderm (Fig. 2b–d). Hence, laminins α1 and α5 are deposited in the VER basement membrane by different tissues, mesoderm and surface ectoderm/VER, respectively.

Considering the laminin β and γ chains, β2, β3, γ1, γ2, and γ3 proteins were all expressed throughout the basement membrane of the ventral tail bud, including the VER (Fig. 2ǵ–ḱ). By contrast, the laminin β1 protein signal was discontinuous around the VER (Fig. 2f́). At the mRNA level, laminins β*3* and γ*2* showed intense signal within the cells of the surface ectoderm, particularly the VER (Fig. 2h, j), γ*1* was expressed mainly in the mesoderm (Fig. 2i), and β*1* transcripts were present in both ectoderm and mesoderm (Fig. 2f). mRNAs for laminins β*2* and γ*3* were not detectable (Fig. 2g, k).

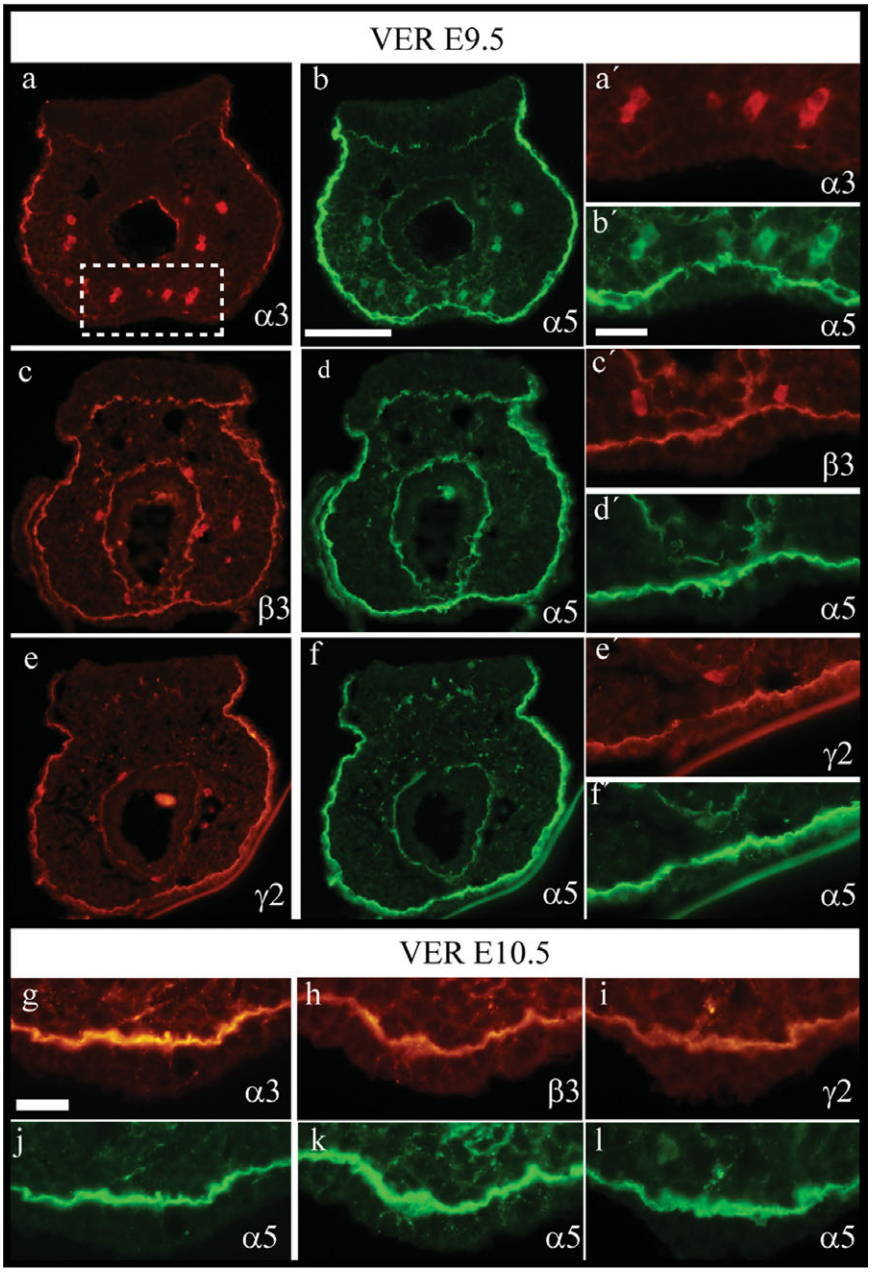

Fig. 3. Co-localization of laminin chains in the basement membrane of the VER. Immunohistochemistry of laminin α5 together with α3 (a, a′, b, b′, g, j), β3 (c, c′, d, d′, h, k), and γ2 (*e, e′, f, f′, l, l′*) on transverse sections of the tail-bud. Embryos were harvested at E9.5 (25–30 somites; a–f, and a′–f′) and E10.5 (40–45 somites; g–l). VER region, as indicated by dotted rectangle in a, is magnified in a′–f′. FITC secondary antibody (green) was used for laminin α5, whereas biotin/streptavidin secondary antibody (red) was used for laminins α3, β3, and γ2. Scale bar in b = 50 μm (applies to a–f) and 20 μm (applies to a'–f' and g–l).

In our previous study of laminin secretion into mouse embryonic basement membranes (Copp et al., 2011), we could infer which of the many possible laminin heterotrimers were present from a comparison of the mRNA and protein distributions of

the different chains. Lamin heterotrimers are assembled intracellularly prior to secretion (Yurchenco et al., 1997; Schneider et al., 2007; Tzu and Marinkovich, 2008) making it obligatory for the mRNAs of the constituent α, β, and γ chains to be co-expressed. In the case of the tail-bud, the predominant laminin chain mRNAs in VER cells were α5, β3, and γ2, with possible minor contributions also from β1 and γ1 (Fig. 2e, h,j). Previously, laminin β3 and γ2 have been described only in combination with α3, as components of laminin 5. Consequently, our results suggest the possibility of a novel laminin heterotrimer, α5β3γ2, expressed by the VER.

To further address the possible existence of a novel laminin isoform in the E9.5 mouse VER, we performed double immunohistochemistry for laminin α5 in combination with laminins α3, β3, or γ2. In the E9.5 tail-bud, the laminin α3 chain was clearly excluded from the basement membrane overlying the VER (Fig. 3a, a', b,b́). In contrast, robust co-labelling of α5 was observed with both laminin β3 (Fig. 3c, c', d,d') and laminin γ2 (Fig. 3e, é, f,f́). Hence, taken together with the very specific expression of mRNAs for these lamin chains in VER cells, we conclude that α5, β3, and γ2 likely form a laminin532 heterotrimer in the basement membrane overlying the VER at E9.5.

At E10.5, double immunostaining also demonstrated co-localisation of laminin α5 with β3 and γ2 throughout the entire surface ectoderm, including the VER (Fig. 3h, i,k, l). By this stage, laminin α3 also co-localised with α5 in the surface ectoderm of the tail-bud (Fig. 3g, j), raising the possibility that two different heterotrimers, α3β3γ2 and α5β3γ2, coexist at this later stage.

Expression of Integrins

Many laminin variants interact with α6 integrins and, with the exception of α1-containing laminins, most also interact to varying degrees with α3 integrins (Barczyk et al., 2010). We therefore assessed the expression of α3 and α6 integrins by immunohistochemistry and in situ hybridization in the mouse embryonic tail bud at E9.5.

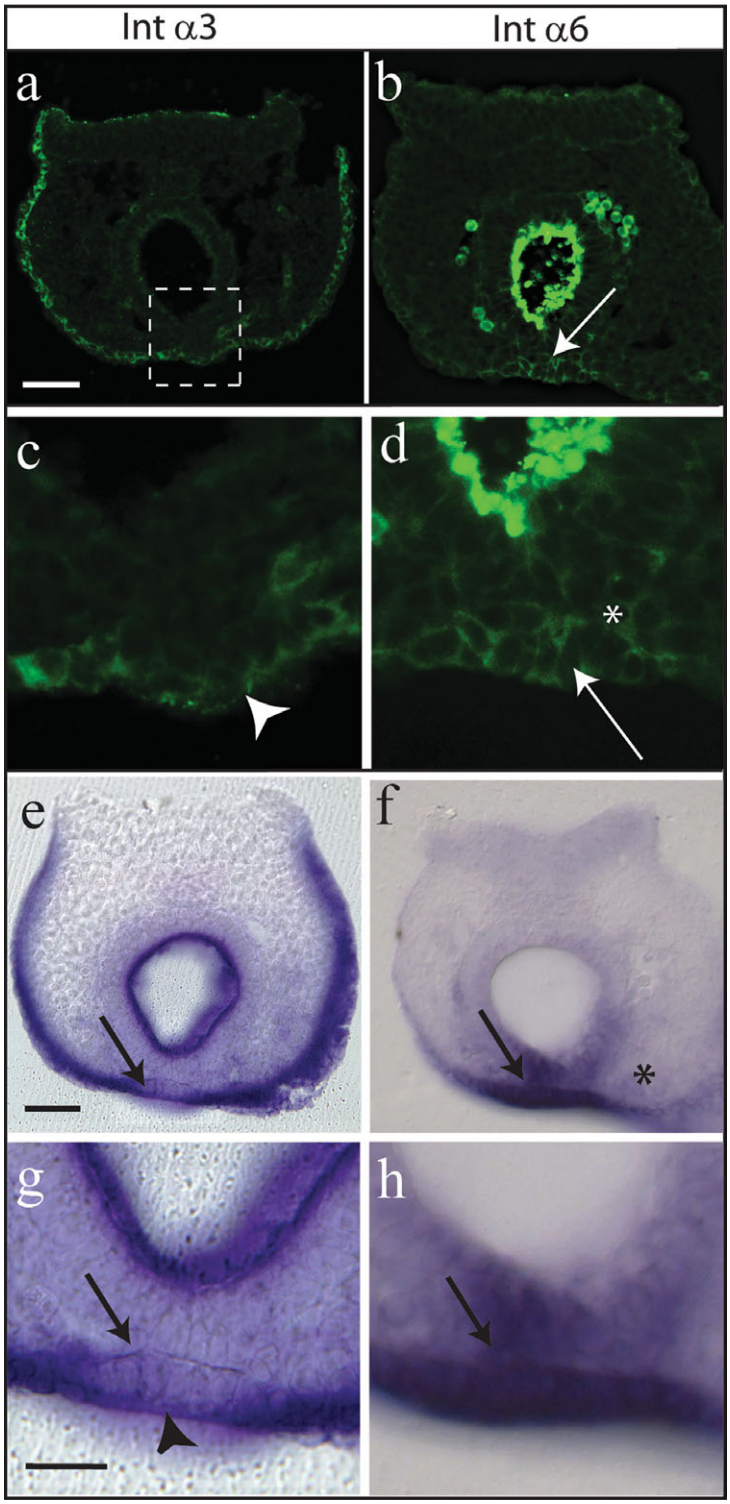

Fig. 4. Expression of α3 and α6 integrins in the VER and surrounding tissues at E9.5. Immunohistochemistry on cryosections (a–d) and mRNA expression on vibratome sections following whole mount in situ hybridisation (e–h) for α3 integrin (a, c,e, g) and α6 integrin (b, d,f, h). High-magnification view of the boxed area in a is shown in c and similar areas are magnified from b, e, and f to obtain panels d, g, and h, respectively. Arrows point to the VER region. Asterisks mark positive α6 signal on VER cell surfaces facing the basement membrane. Arrowheads point to α3 signal solely on the outer facing surfaces of the VER cells. Scale bar in a, e = 50 μm (also in b, f) and in g = 25 μm (applies to c, d, h).

TABLE 1. Primary Antibodies Used in the Immunohistochemistry Studies

Target	Antibody details	Source	References
Integrin α3	Anti-C terminus of chicken integrin α3, 1:100 dilution	Rabbit	DiPersio et al. (1995)
Integrin α6	Anti-human integrin α6, 1:100 dilution	Rat	Abc-serotec, Cambridge, U.K. (MCA699)
Laminin α1	Anti-mouse laminin α1, conditioned medium used undiluted	Rat	Sorokin et al. (1992)
Laminin α2	Anti-mouse laminin α2, conditioned medium used undiluted	Rat	Schuler and Sorokin (1995)
Laminin α3	Anti-α3 IIIa antiserum, 1:500 dilution	Rabbit	Sasaki et al. (2001)
Laminin α4	Anti-α4 LG1 antiserum, 1:500 dilution	Rabbit	Talts et al. (2000)
Laminin α5	Anti-mouse laminin α5, conditioned medium used undiluted	Rat	Sorokin et al. (1997)
Laminin β1	Anti-mouse laminin β1, conditioned medium used undiluted	Rat	Sixt et al. (2001)
Laminin β2	Anti-mouse laminin β2, 1:500 dilution	Rabbit	Agrawal et al. (2006)
Laminin β3	Anti-β3 VI/V antiserum, 1:500 dilution	Rabbit	Sasaki et al. (2001)
Laminin γ1	Anti-γ1, 1:100 dilution	Rat	Chemicon, Billerica, MA (MAB1914)
Laminin γ2	Anti-γ2 antiserum, 1:500 dilution	Rabbit	Sasaki et al. (2001)
Laminin γ3	Anti-γ3 III3-5 antiserum, 1:500 dilution	Rabbit	Gersdorff et al. (2005)

While robust integrin α3 protein and mRNA expression were detected throughout most of the surface ectoderm, inmunostaining was detected solely on the outer facing surfaces of the VER cells (Fig. 4a, c). Consistent with this, mRNA intensity for α3 integrin was markedly reduced in the VER compared with the rest of the surface ectoderm (Fig. 4e, g). In contrast, integrin α6 mRNA expression appeared enhanced specifically in the VER and overlying mesenchyme, whereas it was largely absent from other regions of the surface ectoderm (Fig. 4f, h). Immunostaining indicated the presence of α6 integrin protein on the entire surface (both inner and outer facing) surfaces of the VER cells (Fig. 4b, d). Hence, the expression of integrins α3 and α6 appears complementary in the tail bud at this stage of development, with only α6 integrin being expressed on VER cell surfaces facing the basement membrane.

In the present study, we have found evidence of a putative new laminin variant (laminin532) expressed in the VER, a mid-ventral specialization of the surface ectoderm in the developing mouse tail-bud. Although final confirmation of this novel laminin would require biochemical characterisation, nevertheless, our findings suggest that the α5, β3, and γ2 laminin subunits are co-transcribed in VER cells and then co-deposited in the adjacent basement membrane. Furthermore, our observation that α6 integrin is expressed in the VER provides evidence for a spatially restricted interaction between cells of the VER and laminins within the basement membrane. Although the role of this putative novel laminin variant in the VER remains to be demonstrated, laminins and integrins are well known to be involved in regulation of cell differentiation, for example as demonstrated for laminin α5 in smooth muscle differentiation, and laminin β2 in neuromuscular differentiation (Noakes et al., 1995; Bolcato-Bellemin et al., 2003). We propose, therefore, that laminin532 could interact with α6-containing integrins to direct specialisation of the surface ectoderm during formation of the midline VER.

One of the characteristics of the VER is its involvement in the process of EMT, which is subject to regulation by BMP signalling at this stage of tail-bud development (Ohta et al., 2007). Given the well-described role of laminins and integrins in cell migration (Tzu and Marinkovich, 2008), it is possible that laminin532 could act via integrin α6, in concert with BMP pathway activation, to regulate EMT. Indeed, the laminin γ2 chain is known to undergo proteolytic processing, thereby releasing a fragment (DIII) that binds to the epidermal growth factor receptor (EGFR) to trigger cell migration (Koshikawa et al., 2005). Although most of the work on proteolysis of laminin γ2 has been undertaken with laminin322, it is possible that similar processing could occur with the laminin532 variant. In order to address the role of laminin532 in regulating BMP signalling and EMT in the VER, it will be necessary to explore the effects of interfering directly with the laminin–integrin interactions in the developing tail-bud.

EXPERIMENTAL PROCEDURES

cDNA Probes

First-strand cDNA was prepared using reverse transcriptase (Bioline) with total RNA extracted from E9.5 CD1 mouse embryos using TRIzol reagent (Invitrogen, Carlsbad, CA). A cDNA probe for integrin α3 was prepared using the primers 5′ggtgatgactataccaaccg3′–5′gataaatccc agtccttccg3′, which amplify a 463-bp region located between base pairs 619 and 1,082 (GenBank accession no. BC053031.1). A cDNA probe for integrin α6 was prepared using the primers 5′ccaaggagattagcaatgg3′–5′atctc tcgctcttctttccg3′, which amplify a 620-bp region located between base pairs 2,692 and 3,312 (GenBank accession no. BC058095). PCR products were cloned into pGEMt (Promega, Fichburg, WI) and sequenced to confirm

identity. cDNA probes for *Bmp2, Noggin, Neuralin* (*Chrdl1*), *Cadherin6, Msx1, Msx2, RhoB, and laminins* α*1,* α*2,* α*3,* α*4,* α*5,* β*1,* β*2,* β*3,* γ*1,* γ*2*, and γ*3* are described elsewhere (Henderson et al., 2000; Ybot-Gonzalez et al., 2007; Copp et al., 2011).

In Situ Hybridisation

Whole mount in situ hybridisation was carried out using sense and antisense digoxygenin-labelled riboprobes prepared using a digoxigenin RNA labelling kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. E9.5 (25–30 somites) mouse embryos were washed twice in DEPC-treated PBS and fixed overnight in 4% paraformaldehyde. To reveal sites of mRNA expression, embryos were processed as described previously (Ybot-Gonzalez et al., 2005). Selected embryos, labelled with individual probes, were embedded in a gelatine-sucrose-albumin (1:1.5:60) solution, solidified by addition of 2.5% of glutaraldehyde. A vibratome was used to obtain 50-μm sections, which were mounted with 50% glycerol, and photographed with an Axiophot (Zeiss, Thornwood, NY) photomicroscope. Sense-strand riboprobes used as a control for specificity gave no specific signal.

Immunohistochemistry

The method was as described previously (Copp et al., 2011), but with variations as follows. E9.5 (25–30 somites) and E10.5 (35–40 somites) mouse embryos were fixed overnight in either 4% paraformaldehyde in PBS at 4°C, for immunostaining with anti-integrin α6 or laminin α2 antibodies, or in zinc fixative (3 mM calcium acetate, 0.023 M zinc acetate, 0.036 M zinc chloride in 0.1 M Tris buffer, pH 7.4) at room temperature and overnight, for anti-integrin α3 and all other anti-laminin antibodies. Prior to blocking, sections for anti-laminin staining were treated with 0.05% hyaluronidase in PBS for 2 hr at 37°C. Primary antibodies are shown in Table 1. Secondary antibodies were: goat anti-rat FITC conjugated (Abcam, Cambridge, UK; ab97056), goat anti-rat Biotin conjugated (Sigma, St. Louis, MO; B7139), goat anti-rabbit Biotin conjugated (Life Technologies, Carlsbad, CA; 65–6,140), and goat anti-rabbit FITC conjugated, Abcam (ab6717), all used at 1:250 dilution. Streptavidin was obtained from Sigma (S6402). Representative sections were selected from at least three serially sectioned embryos of each staining type, and photographed with an Olympus (Tokyo, Japan) BX-61 photomicroscope.

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